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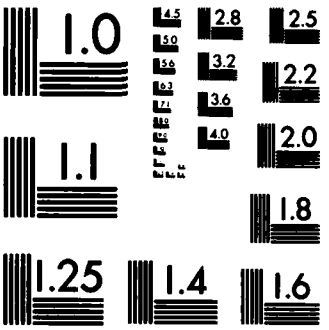

MICROCOPY RESOLUTION TEST CHART
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The time courses of synthesis of three neuronal cell-type specific phosphoproteins, Synapsin I, Synapsin III and G-Substrate, were determined in developing rat brains. The appearance of these proteins was concurrent with synapse formation. Two partial cDNA clones for Synapsin I were isolated from a rat brain cDNA expression library and the nucleotide sequences were determined. The mRNA for these clones was shown to be brain specific. Estimates for the sizes of the potential mRNAs for Synapsin I and Synapsin III were determined. A second rat brain cDNA expression library was generated for screening for synapsin III clones. A monoclonal antibody specific for synapsin III was identified for screening this library. A bovine caudate cDNA and rabbit cerebellar cDNA library were generated for obtaining, respectively, DARPP-32 and G-Substrate cDNA clones. Oligonucleotide probes specific for DARPP-32 and G-Substrate were synthesized for screening these libraries.

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1. SUMMARY - ABSTRACT OF TECHNICAL PROGRESS

The time courses of synthesis of three neuronal cell-type-specific phosphoproteins, Synapsin I, synapsin III and G-Substrate, were determined in developing rat brains. The appearance of these proteins was concurrent with synapse formation. Two partial cDNA clones for Synapsin I were isolated from a rat brain cDNA expression library and the nucleotide sequences were determined. The amino acid sequences determined from these clones exhibited a high degree of homology with the amino acid sequences derived from the analysis of peptide fragments of bovine Synapsin I. The mRNA for these clones was shown to be brain specific. An oligonucleotide specific for the 5' end of one of these clones was synthesized and used as a primer for generating cDNA libraries enriched for Synapsin I clones. Estimates for the sizes of the potential mRNAs for Synapsin I and synapsin III were determined. A second rat brain cDNA expression library was generated for screening for synapsin III clones. A monoclonal antibody specific for synapsin III was identified for screening this library. A bovine caudate cDNA and rabbit cerebellar cDNA library were generated for obtaining, respectively, DARPP-32 and G-Substrate cDNA clones. Oligonucleotide probes specific for DARPP-32 and G-Substrate were synthesized for screening these libraries.

Direct injection experiments have demonstrated that Synapsin I regulates neurotransmitter release from nerve terminals. Synapsin I and calcium/calmodulin-dependent protein kinase II were pressure-injected into the preterminal digit of the squid giant synapse to test directly the possible regulation of neurotransmitter release by these substances. Neurotransmitter release was determined by measuring the amplitude, rate of rise, and latency of the postsynaptic potential generated in response to presynaptic depolarizing steps under voltage clamp conditions. Injection of dephosphosynapsin I decreased the amplitude and rate of rise of the postsynaptic potential, whereas injection of either phosphosynapsin I or heat-treated dephosphosynapsin I was without effect. Conversely, injection of calcium/calmodulin-dependent protein kinase II, which phosphorylates Synapsin I on site II, increased the rate of rise and amplitude and decreased the latency of the postsynaptic potential. A Synapsin I-like protein and calcium/calmodulin-dependent protein kinase II were demonstrated by biochemical and immunochemical techniques to be present in squid nervous tissue. The data support the hypothesis that Synapsin I regulates the availability of synaptic vesicles for release; it is proposed that calcium entry into the nerve terminal activates calcium/calmodulin-dependent protein kinase II, which phosphorylates Synapsin I on site II, dissociating it from the vesicles and thereby removing a constraint in the release process.

2. RESEARCH OBJECTIVES

The purpose of this research is to attempt to increase the bioreactivity of the nervous system by modifying synaptic transmission at the biochemical level. Since protein phosphorylation appears to be directly involved in synaptic transmission, this study focuses on those aspects of the protein phosphorylation system which are susceptible to external manipulations. Various conditions which alter neuronal function will be studied for their effect on the following parameters of protein phosphorylation (a) transcription of mRNA specific for selected protein kinases and substrate phosphoproteins and (b) synthesis of these selected protein kinases and substrate phosphoproteins by translation of the specific mRNAs.

The specific objectives are (a) to develop brain-specific cDNA libraries, (b) to isolate molecular clones for selected specific protein kinases and substrate phosphoproteins, (c) to determine the nucleotide and peptide sequences of these kinases and phosphoproteins, (d) to determine the normal time course for the expression of the mRNAs coding for these proteins (transcription) in the developing brain, (e) to determine the normal time course for the expression of these proteins (translation) in the developing brain, (f) to determine which factors affect the expression of these proteins in the normal adult brain and, (g) to determine which external factors can affect the expression of these proteins.

3. STATUS OF THE RESEARCH

MOLECULAR CLONING OF cDNA FOR NEURONAL PHOSPHOPROTEINS

Total poly A+ RNA was isolated from total rat brain by homogenization in guanidine thiocyanate, centrifugation through CsCl and chromatography on oligo dT cellulose. cDNA was synthesized by reverse transcription and ligated into the expression vector pUR250 with EcoR1 and BamH1 linkers by standard procedures. The colonies were screened for inserts coding for Synapsin I with rabbit serum antibodies specific for Synapsin I and horseradish peroxidase-conjugated goat anti-rabbit IgG serum. Of the 6000 colonies screened, four colonies were positive for Synapsin I synthesis in both the primary and secondary screens. A total protein lysate from each of the four positive colonies was prepared, separated on SDS-polyacrylamide gels and blotted on nitrocellulose paper. The blots were incubated separately with three preparations of rabbit serum antibodies and five preparations of monoclonal antibodies specific for Synapsin I. The antibodies bound to different molecular weight bands for each of the clones (Clone #1: 12,000 Daltons; Clone #2: 30,000 Daltons; Clone #3: 35,000 Daltons; Clone #4: 40,000 Daltons). All preparations of rabbit serum antibodies and monoclonal antibodies specific to Synapsin I bound to Clone #4. No more than three of the four serum antibody preparations bound to the other three clones. These observations are consistent with the interpretation that these four clones contain inserts for Synapsin I cDNA and are expressing the protein. Clone #4 contains an insert coding for a major portion of the Synapsin I molecule (57,000 Daltons). Clones #1,2 and 3 contain smaller inserts which do not contain all of the antigenic sites recognized by our serum antibodies. Total RNA and poly A+ RNA from total rat brain, partially purified Synapsin I mRNA, and total RNA and poly A+ RNA from lung and spleen were separated on denaturing agarose gels and blotted onto nitrocellulose paper. The nitrocellulose paper was incubated with nick-translated DNA labelled with [^{32}P]-dCTP generated with plasmid DNA from Clone #4. The probe bound to three bands of 2.7, 1.8 and 1.0 kilobases on the rat brain RNA blot. Only one band, the 2.7 kilobase band, was visible on the blot of partially purified Synapsin I mRNA. No bands were visible on the blots of lung and spleen RNA, although these blots did bind the actin control probe. These results are consistent with the interpretation that the mRNA coding for Synapsin I is represented by the 2.7-kilobase band. The 1.8-and 1.0-kilobase bands are of sufficient size to represent the mRNA's coding for, respectively, synapsin IIIa and synapsin IIIb. The fact that the Clone #4 probe also binds to these bands is consistent with previous observations that there may be homologies between Synapsin I and synapsin III. The nucleotide sequences of Clone #4 and Clone #2 were determined by the dideoxy sequencing method. The derived peptide sequence of Clone #4 is:

5' gly ser arg arg arg gly pro pro leu val ala pro arg pro leu
arg ala leu ala ala leu arg pro ser pro lys gln met met asn
ser glu ala arg leu ser asp 3'

The sequence of Clone #4 was compared with the fragments of sequence for Synapsin I determined by peptide sequencing to confirm that Clone #4 contains an insert coding for Synapsin I. The sequence of Clone #2 was contained within the sequence of Clone #4. Hybrid selection, coupled with in vitro translation, is being carried out to confirm that these clones code

for Synapsin I. Three more cDNA libraries, enriched for 3' ends, have been generated to isolate a clone containing cDNA for the extreme 3' region of Synapsin I. An oligonucleotide specific for the 5' end of Clone #4 was synthesized and used as a primer to generate cDNA libraries enriched for the 5' region of Synapsin I. RNA from a wide range of tissues (e.g. liver, kidney, spleen, heart, lung) has been isolated to further demonstrate the brain specificity of these clones by Northern blots. Total rat DNA has been isolated to determine the number of genes coding for Synapsin I (and the other phosphproteins) by Southern blots.

A rat brain cDNA expression library has been generated for screening for molecular clones of synapsin III. Five lines of monoclonal antibodies generated against Synapsin I and three lines of monoclonal antibodies generated against synapsin III were tested for cross-reactivity. At least one of the monoclonal antibodies generated against synapsin III was of sufficient specificity to be used for screening the library for clones of synapsin III. This screening is currently in progress. RNA was isolated from adrenal medullas and chromaffin cells grown in culture (both of which contain synapsin III, but not Synapsin I), for isolating RNA for generating libraries containing clones for synapsin III and for use in identifying putative synapsin III cDNA clones obtained from rat brain cDNA libraries.

Libraries of bovine caudate cDNA and rabbit cerebellar cDNA were generated. These libraries are being screened with synthetic oligonucleotide probes with sequences that match the potential sequences coding for DARPP-32 and G-substrate. These sequences were derived from the sequences of fragments of DARPP-32 and G-substrate determined by peptide sequencing.

DEVELOPMENTAL BIOLOGY OF THE SYNTHESIS OF NEURONAL PHOSPHOPROTEINS

Brains were dissected from rats of various ages from birth to adult and immediately frozen in liquid nitrogen. Cytosolic polysomes from rat brain exclusive of cerebellum were isolated by homogenization and centrifugation through a sucrose shelf. The isolated polysomes were used to direct protein synthesis in a rabbit reticulocyte in vitro translation system in the presence of ^{35}S-methionine. Serum antibodies specific for Synapsin I and synapsin III were added to the translation mixture and precipitated out of solution by the addition of Staphylococcus aureus cells. The protein products were separated by SDS-polyacrylamide gel electrophoresis and subjected to autoradiofluorography. The bands corresponding to Synapsin I and synapsin III were cut from the gel and the incorporated ^{35}S was measured by a scintillation counter. The degree of Synapsin I and synapsin III synthesis correlated well with the amount of synaptogenesis occuring during each developmental stage.

Cytosolic polysomes from the cerebella of rats were isolated and used to direct in vitro translation as described above. Serum antibodies specific for G-substrate were added to the translation mixture and precipitated out of solution by the addition of Staphylococcus aureus cells. The protein products were separated by SDS-polyacrylamide gel electrophoresis and subjected to autoradiofluorography. The band corresponding to G-substrate was scanned with a densitometer and the values for the G-substrate peaks were normalized against the value for postnatal

day 20. Synthesis of G-substrate could not be detected before postnatal day 6. The level of synthesis increased slowly from postnatal day 6 to 12, increased rapidly from postnatal day 12 to 24, then decreased to the adult level. There was no correlation between the relative level of G-substrate synthesis and the relative total protein-synthesizing activity of the cerebellar polysomes during development. The period of G-substrate synthesis was concurrent with the period of formation of synapses between Purkinje cells and the other neuronal cells of the cerebellum. The observation that the synthesis of G-substrate occurred late in the development of Purkinje cells supports the interpretation that this phosphoprotein is involved in a function characteristic of these specialized cells.

4. PUBLICATIONS

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Walaas, S. I., Ouimet, C. C., Hemmings, H. C., Jr. & Greengard, P. (1984) DARPP-32, a dopamine regulated phosphoprotein in the basal ganglia. In: Dopamine Systems and Their Regulation. (G. D. Woodruff, ed.) MacMillan Press. In press.

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Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1985) Intraterminal injection of synapsin I or of calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc. Natl. Acad. Sci. U.S.A. In press.

5. PROFESSIONAL PERSONNEL

Members of the Laboratory of Molecular and Cellular Neuroscience at the Rockefeller University who are associated with this research effort include:

Dr. Paul Greengard, Professor and Laboratory Head

Dr. Robert M. Lewis, Postdoctoral Fellow

Dr. Selma Kanazir, Research Associate

Dr. Ken Mackie, Postdoctoral Fellow

Dr. Louis DeGennaro, Guest Investigator

Dr. Teresa McGuinness, Guest Investigator

6. INTERACTIONS

Papers presented at scientific meetings:

Walaas, S. I., Hemmings, H. C., Jr., Ouimet, C. C. & Greengard, P. (1984) DARPP-32, a dopamine- and cyclic AMP-regulated phosphoprotein in brain. In Fifth International Conference on Cyclic Nucleotides and Protein Phosphorylation, Volume 17A of Advances in Cyclic Nucleotide Research (P. Greengard and G. A. Robison, series editors), Raven Press, New York.

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